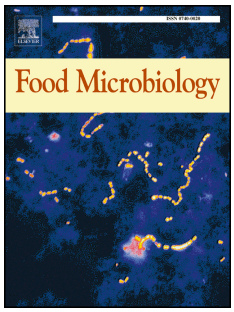
Accepted Manuscript



Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7

Alicia Campion, Ruth Morrissey, Des Field, Paul D. Cotter, Colin Hill, R. Paul Ross

PII: S0740-0020(16)30065-X

DOI: 10.1016/j.fm.2017.01.020

Reference: YFMIC 2729

To appear in: *Food Microbiology*

Please cite this article as: Alicia Campion, Ruth Morrissey, Des Field, Paul D. Cotter, Colin Hill, R. Paul Ross, Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7, *Food Microbiology* (2017), doi: 10.1016/j.fm.2017.01.020

Highlights

- Bioengineered nisin variants and essential oils were tested for inactivation of C. sakazakii and E. coli O157:H7.
- Nisin variant and essential oil combinations caused extended lag phases of growth compared to nisin A-essential oil combinations.
- Nisin variant-carvacrol combinations significantly reduced C. sakazakii and E. coli O157:H7 compared to nisin A-carvacrol treatment.
- Nisin variant-carvacrol combinations caused complete inactivation of E. coli O157:H7 in apple juice compared to nisin A-carvacrol treatment.
- Commercial Nisaplin and citric acid combinations also resulted in complete inactivation of C. sakazakii in infant formula.

Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control *Cronobacter sakazakii* and *Escherichia coli* O157:H7

Alicia Campion [a], Ruth Morrissey [a,b], Des Field [a], Paul D. Cotter [b,c,*], Colin Hill [a,b,*] and R. Paul Ross [b,d]

[a] School of Microbiology, University College Cork, Cork, Ireland

[b] APC Microbiome Institute, University College Cork, Cork, Ireland

[c] Teagasc, Moorepark Food Research Centre, Fermoy, Co. Cork, Ireland

[d] College of Science, Engineering and Food Science, University College Cork, Cork, Ireland

*Corresponding authors. Tel: +353 25 42694/ +353 21 490 1373

E-mail addresses: paul.cotter@teagasc.ie (P. D. Cotter); c.hill@ucc.ie (C. Hill).

Abstract

Cronobacter sakazakii and *Escherichia coli* O157:H7 are well known food-borne pathogens that can cause severe disease. The identification of new alternatives to heating to control these pathogens in foods, while reducing the impact on organoleptic properties and nutritional value, is highly desirable. In this study, nisin and its bioengineered variants, nisin V and nisin S29A, are used alone, or in combination with plant essential oils (thymol, carvacrol and trans-cinnamaldehyde) or citric acid, with a view to controlling *C. sakazakii* and *E. coli* O157:H7 in laboratory-based assays and model food systems. The use of nisin variants (30 µM) with low concentrations of thymol (0.015%), carvacrol (0.03%) and trans-cinnamaldehyde (0.035%) resulted in extended lag phases of growth compared to those for corresponding nisin A-essential oil combinations. Furthermore, nisin variants (60 µM) used in combination with carvacrol (0.03%) significantly reduced viable counts of *E. coli* O157:H7 (3-log) and *C. sakazakii* (4-log) compared to nisin A-carvacrol treatment. Importantly, this increased effectiveness translated into food. More specifically, sub-inhibitory concentrations of nisin variants and carvacrol caused complete inactivation of *E. coli* O157:H7 in apple juice within 3 hours at room temperature compared to that of the equivalent nisin A combination. Furthermore, combinations of commercial Nisaplin and the food additive citric acid reduced *C. sakazakii* numbers markedly in infant formula within the same 3 h period. These results highlight the potential benefits of combining nisin and variants thereof with carvacrol and/or citric acid for the inhibition of Gram negative food-borne pathogens.

Keywords

C. sakazakii, *E. coli* O157:H7, Nisin, Essential oils, Apple juice, Infant formula milk

1. Introduction

Cronobacter sakazakii and *Escherichia coli* O157:H7 are both significant Gram negative foodborne pathogens. They have garnered special notoriety because of their association with life-threatening diseases. Their presence in food poses a serious health risk for consumers and is a safety concern for the food industry. Enterohaemorrhagic *E. coli* O157:H7 can cause devastating and severe illness such as haemorrhagic colitis and haemolytic uremic syndrome. Approximately 10-15% of *E. coli* O157:H7 infections result in haemolytic uremic syndrome, causing acute renal failure in children and 3-5% of cases are fatal (Ho et al., 2013). There have been several outbreaks associated with the consumption of food contaminated with *E. coli* O157:H7 (Vidovic and Korber, 2014). Similarly, *C. sakazakii* can cause a range of serious neonatal infections such as meningitis, septicaemia and enteritis (Drudy et al., 2006; Gurtler et al., 2005). Several disease outbreaks have been associated with the contamination of powdered infant formula milk (CDC, 2002; Iversen and Forsythe, 2004). *C. sakazakii* has a high mortality rate of 40-80%, and death can occur within hours (Bowen and Braden, 2006; Norberg et al., 2012). Infection may also result in severe sequelae such as hydrocephalus, quadriplegia and retarded neural development among survivors (Forsythe, 2005).

Heat treatments and chemical preservatives are commonly used as hurdles to control foodborne pathogens and spoilage bacteria. However, these processes may have undesirable effects, such as altering the nutritional and sensory properties of the food. Furthermore, there has been an increasing consumer demand for additive-free, minimally processed foods, while still maintaining adequate microbiological safety and stability. Therefore, the use of natural antimicrobials as food preservatives has been the focus of ever-increasing attention. Among these natural alternatives are bacteriocins. Bacteriocins are ribosomally synthesised, post-translationally modified peptides that are produced by bacteria and which are active against other bacteria. They can have a narrow range of activity within their own species or a broad spectrum of activity across genera (Cotter et al., 2005). Although there are numerous bacteriocins with food preservation potential, only nisin, produced by *Lactococcus lactis*, is used extensively. Nisin A has been assigned to the lantibiotic class of bacteriocins due to the presence of unusual amino acids that arise due to the post-translational modification of serine and threonine residues ultimately leading to the formation of lanthionine and β-methyllanthionine ring structures, respectively (Bierbaum and Sahl, 2009; Sahl et al., 1995). Nisin A is used in over 50 countries worldwide and has been approved for use by both the EU (E234) and the Food and Drug Administration (FDA) (Delves-Broughton, 1990). Nisin A functions through a unique dual mode of action. It binds to lipid II, an essential precursor to cell wall biosynthesis, while also inserting itself into the bacterial cell membrane. This facilitates pore formation and ultimately leads to the loss of solutes from the bacterial cell resulting in cell death (Wiedemann et al., 2004; Wiedemann et al., 2001).

The gene-encoded nature of nisin A allows for its manipulation in order to modify its biological and physical properties. Indeed, recent research has shown that bioengineering of nisin A can result in variants with greater potency towards food-borne pathogens (Field et al., 2015b). One particular variant, M21V (nisin V), has shown enhanced activity towards several Gram positive pathogens, including *Listeria monocytogenes*, compared to that of nisin A (Field et al., 2010). Although nisin A is effective against Gram positive bacteria such as *Staphylococci, Bacilli* and *Clostridia* (Bierbaum and Sahl, 2009; Sobrino-López and Martín-Belloso, 2008), Gram negative bacteria are generally not as sensitive. However, novel variants, such as nisin S29A and S29G, with enhanced activity towards Gram negative food-associated pathogens exist (Field et al., 2012). Nisin A may also be effective against Gram negatives if their outer membrane is destabilized with chelating agents (Stevens et al., 1991). Membrane disruption/permeabilisation is also thought to be the basis for the observation that nisin, when combined with the phenolic compounds carvacrol and thymol which possess membrane permeability properties, exhibit enhanced activity against Gram negative bacteria by permitting nisin to pass through the protective outer membrane (Helander et al., 1998). In fact, there are several studies demonstrating that nisin and essential oil combinations exhibit enhanced inhibitory effects against both Gram positive and Gram negative bacteria (Ettayebi et al., 2000; Olasupo et al., 2003; Olasupo et al., 2004; Periago and Moezelaar, 2001; Pol and Smid, 1999; Yuste and Fung, 2004). More recently, nisin-containing semi-purified preparations in combination with carvacrol and trans-cinnamaldehyde were established to more effectively inhibit *L. monocytogenes* than either treatment alone (Field et al., 2015a).

The aim of this study was to evaluate the antimicrobial activity of nisin A, or the bioengineered nisin derivatives nisin V and S29A, when combined with the essential oils, thymol, carvacrol or trans-cinnamaldehyde, or citric acid against the Gram negative pathogens *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} or *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}.

2. Materials and Methods

2.1 Bacterial strains and growth conditions

The nisin producing strains and *lux*-tagged bacterial strains used in this study are listed in Table 1. *L. lactis* strains were grown in M17 broth (Oxoid) supplemented with 0.5% glucose (GM17) or GM17 agar at 30°C. *E. coli* and *C. sakazakii* cultures were grown in Luria-Bertani (LB) broth or agar at 37°C. When required, antibiotics were used where indicated at the following concentrations: chloramphenicol at 10 μg ml^{-1} for *L. lactis* and erythromycin at 500 μg ml^{-1} for *E. coli* and *C. sakazakii*. Stock solutions of thymol (Sigma) were prepared at 50 mg ml^{-1} in 50% ethanol and stored

143 at -20°C. Carvacrol and trans-cinnamaldehyde (Sigma) were diluted from stock
144 (0.976 g ml^{-1} and 1.05 g ml^{-1}, respectively) in 50% ethanol to the desired
145 concentration. Stock solutions of Nisaplin (Sigma) and citric acid (Sigma) were
146 prepared at 100 mg ml^{-1} and 500 mM in sdH_20, respectively, filter sterilised and
147 diluted to the desired concentration. In all experiments, the concentration of ethanol
148 did not exceed 2% (vol/vol).
149
150 *2.2 Nisin purification*
151
152 Purification of wild type nisin A and nisin derivatives were carried out as
153 described previously (Field et al., 2010). Briefly, overnight cultures of nisin producing
154 strains were grown in GM17 broth at 30°C and were subsequently inoculated into
155 two litres of purified TY broth at 1% and incubated overnight at 30°C. The culture
156 was centrifuged at 7,000 r.p.m. for 20 minutes and the supernatant retained. The
157 cell pellet was resuspended in 300ml of 70% isopropanol 0.1% TFA and magnetically
158 stirred for 3 h at room temperature. Cell debris was removed by centrifugation at
159 7,000 r.p.m. for 20 minutes and the supernatant retained. The supernatant was
160 applied to a 60 g Amberlite bead (Sigma) column, which was subsequently washed
161 with 500 ml of 30% ethanol and the inhibitory activity eluted in 500 ml of 70%
162 isopropanol 0.1% trifluoroacetic acid (TFA). The isopropanol was evaporated off
163 using a rotary evaporator (Buchi) to a volume of 160ml and the sample pH adjusted
164 to approximately 4.2. The sample was applied to a 10g (60ml) Varian C-18 Bond Elut
165 Column previously pre-equilibrated with HPLC water and methanol. The column was
166 washed with 120 ml of 30% ethanol and the inhibitory activity eluted in 60 ml of 70%

isopropanol 0.1% TFA. Six millilitres of the lantibiotic preparation was concentrated to 1 ml through the removal of the isopropanol by rotary evaporation and applied to a Phenomenex C12 reverse-phase (RP)-HPLC column, previously equilibrated with 25% isopropanol 0.1% TFA. The column was then developed in a gradient of 30% isopropanol 0.1% TFA to 60% isopropanol 0.1% TFA from 10 to 45 minutes at a flow rate of 2.1 ml min^{-1}. Fractions containing nisin A and nisin derivative peptides were collected and subjected to Mass Spectrometry with a Shimadzu Biotech MALDI-TOF Mass Spectrometer (AXIMA-CFR plus model).

2.3 Mass spectrometry

For colony mass spectrometry analysis, bacteria were collected with sterile plastic loops and mixed with 50 ml of 70% isopropanol adjusted to pH2 with HCl. The suspension was vortexed, centrifuged at 14,000 r.p.m. for 2 minutes and the supernatant retained for analysis. Mass spectrometry in all cases was performed with an Axima CFR plus matrix-assisted laser desorption/ionisation time-of-flight (MALDI TOF) mass spectrometer (Shimadzu Biotech, Manchester, UK.) A 0.5 µl aliquot of matrix solution (alpha-cyano-4-hydroxy cinnamic acid (CHCA), 10mg ml^{-1} in 50% acetonitrile-0.1% (v/v) trifluoroacetic acid) was placed onto the target and left for 1-2 minutes before being removed. The residual solution was then air dried and the sample solution (re-suspended lyophilized powder or colony mass spectrometry supernatant) was positioned onto the pre-coated sample spot. Matrix solution (0.5 ml) was added to the sample and allowed to air dry. The sample was subsequently analysed in positive-ion reflectron mode.

191

192

193 *2.4 Minimum inhibitory concentration (MIC) assays*

194

195 The MIC of nisin peptides against Gram negative organisms were carried out
196 in triplicate in microtitre plates as previously described (Field et al., 2010). Briefly,
197 96-well microtitre plates were pre-treated with phosphate buffered saline (PBS)
198 containing 1% (w/v) bovine serum albumin (BSA) and subsequently incubated at
199 37°C for 30 minutes. Wells were washed with PBS and allowed to air-dry before the
200 addition of 100 µl double-strength LB broth. Gram negative strains were grown
201 overnight in LB broth at 37°C, subcultured into fresh LB broth and grown to mid-
202 logarithmic phase (OD_{600nm} ~ 0.5). The cells were harvested by centrifugation,
203 washed with 10 mM SPB at pH 7.4 and diluted to 1 $\times 10^5$ cfu ml^{-1} in 10 mM SPB pH
204 7.4. Nisin and nisin derivative purified peptides were resuspended in double-
205 strength LB broth to a stock concentration of 60 µM. Peptides were then adjusted to
206 a starting concentration of 15 µM and 2-fold dilutions of the nisin peptides were
207 carried out in the 96-well plates. Target organisms were added and plates were
208 incubated at room temperature overnight (~16 h). The MIC was taken as the lowest
209 concentration of peptide at which growth was inhibited. The MIC of essential oils
210 against Gram negative strains were carried out as above but with minor variations;
211 BSA treatment of 96-well plates were not required and essential oils were diluted to
212 a starting concentration of 2 mg ml^{-1} for serial dilution of thymol, carvacrol and
213 trans-cinnamaldehyde.

214

2.5 Growth/Kill assays

Growth and kill assays were carried out using representative strains as a consequence of the limited amount of pure material available. For growth assays, overnight cultures of target strains were harvested by centrifugation, washed in 10 mM SPB pH 7.4 and transferred (1×10^7 cfu ml^{-1} in a 1.0 ml volume) into LB broth containing nisin purified peptide alone and in combination with one of the essential oils being investigated. A volume of 200 µl was transferred to a 96-well plate (Genetix) and cell growth was measured spectrophotometrically using a Spectra Max 340 spectrophotometer (Molecular Devices, Sunnyvale, California) for 24 h. For kill assays, overnight cultures of target strains were again harvested by centrifugation, washed in 10 mM SPB pH 7.4 and transferred (1×10^7 cfu ml^{-1} in a 0.5 ml volume) into LB broth containing nisin purified peptide alone and in combination with one of the essential oils. Samples were incubated for 3 h at room temperature before serial dilution and enumeration on LB agar plates. All experiments were carried out in triplicate.

2.6 Infant milk formula trial

Commercially available powdered infant formula (PIF) (Aptamil™ First Milk) was prepared according to manufacturer's instructions and brought to room temperature. The pH of the reconstituted PIF was 6.8 as determined with a pH meter. An overnight culture of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} was washed in 10 mM SPB pH 7.4, diluted and inoculated into reconstituted PIF at a final

239 concentration of 1 × 10^5 cfu ml^{-1}. PIF samples were treated with 201.12 µg ml^{-1} (60
240 µM) of nisin A, nisin V or nisin S29A, alone and in combination with carvacrol at a
241 concentration of 300 µg ml^{-1}. Samples with carvacrol alone, *C. sakazakii* NCTC
242 8155::p16S*lux*-P_{help} alone and PIF alone served as controls. Samples were incubated
243 at room temperature and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} levels were
244 determined through serial dilution and plate count technique on Druggan-Forsythe-
245 Iversen (DFI) agar at 3 h. Where Nisaplin (Sigma), and citric acid (Sigma) were
246 employed, concentrations of 10 mg ml^{-1} and 30 mM were used, respectively.
247 Nisaplin (containing 2.5% nisin) was resuspended in sdH_2O and filter sterilised before
248 use. The addition of citric acid did not significantly alter the pH of PIF. All
249 experiments were carried out in triplicate.
250
251 *2.7 Apple juice trial*
252
253 Commercially available apple juice was brought to room temperature,
254 filtered and the pH determined as 3.2 with a pH meter. An overnight culture of *E. coli*
255 O157:H7 TUV 93-0::p16S*lux*-P_{help} was washed in 10 mM SPB pH 7.4, diluted and
256 inoculated into the apple juice at a final concentration of 1 × 10^5 cfu ml^{-1}. Apple juice
257 samples were treated with 100.56 µg ml^{-1} (30µM) of nisin A, nisin V or nisin S29A,
258 alone and in combination with carvacrol at a concentration of 75 µg ml^{-1}. Samples
259 with carvacrol alone, *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} alone and apple juice
260 alone served as controls. Samples were incubated at room temperature and *E. coli*
261 O157:H7 TUV 93-0::p16S*lux*-P_{help} levels were determined through serial dilution and

plate count technique on Sorbitol MacConkey agar at 3 h. All experiments were carried out in triplicate.

2.8 Statistical analysis

CFU data was transformed to $\log_{10}$ prior to analysis using the statistical software package GraphPad Prism 6. All comparisons were based on the mean ± standard deviation. Statistical significance was determined via GraphPad prism t-test. In all cases, a P value less than 0.05 were considered to be statistically significant.

Parametric data was analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Non-parametric data was analysed using the Kruskal-Wallis one way ANOVA followed by Dunn's multiple comparisons test. Asterisks rating of *, **, *** or **** indicates statistically significant differences between groups (P≤0.05, P≤0.01, P≤0.001, P≤0.0001, respectively).

3. Results

3.1 Minimum inhibitory concentration assays

Nisin V and nisin S29A are previously generated nisin derivatives that exhibit enhanced activity against a number of targets arising from single amino acid alterations (Fig. 1) (Field et al., 2012). These peptides and nisin A were purified and, following purification and freeze-drying, mass spectrometry analysis was performed.

Peptide masses of 3,321 Da, 3,336 Da and 3,353 Da were obtained for nisin peptides V, S29A and nisin A as expected (data not shown). To more accurately quantify the specific activity of the peptides, broth-based MIC determination assays were carried out using purified peptides against the chosen Gram negative targets (Table 2). Nisin S29A exhibited two-fold greater specific activity than nisin A against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} (3.75 μM and 7.5 μM, respectively) and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (1.87 μM and 3.75 μM, respectively). In the case of nisin V, enhanced specific activity compared to nisin A was observed for *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} (3.75 μM and 7.5 μM, respectively) but activity was equal to that of nisin A when examined against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}. These results demonstrate for the first time the enhanced activity of nisin V against a Gram-negative strain (*E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}) in a broth-based MIC assay. The enhanced efficacy of S29A compared to nisin A against some Gram negative strains has previously been reported (Field et al., 2012).

The susceptibility of the Gram-negative strains to the essential oils thymol, carvacrol and trans-cinnamaldehyde was also assessed in order to ascertain appropriate concentrations for combinatorial assays (Table 2). The essential oils were found to be inhibitory at a concentration of 250 μg ml^{-1} against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}, with the exception that thymol prevented the growth of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} at a concentration of 125 μg ml^{-1}. These values are consistent with those previously reported (Hyldgaard et al., 2012).

3.2 Growth and kill-curve assays

Having shown the increased specific activity of the nisin variants against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}, a more detailed investigation of the impact of the nisin peptides alone, and in combination with essential oils, on bacterial growth was examined. Due to relatively large inoculum (10^7 cfu ml^{-1}) employed for growth curve analysis, the impact of concentrations of 30 µM and 15 µM nisin peptides against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}, respectively, was tested along with varying concentrations (75-200 µg ml^{-1}) of essential oils. When nisin V was used in combination with 100 µg ml^{-1} thymol and 125 µg ml^{-1} carvacrol or trans-cinnamaldehyde, a more profound delay in growth was observed compared to that of either treatment alone against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (Figs. 2). Nisin S29A-essential oil combinations were also better than their nisin A-essential oil equivalents (Fig. 2). However, statistically significant differences in bacterial inhibition were recorded only when nisin variants (V and S29A) were used in combination with the essential oil carvacrol and trans-cinnamaldhyde (Figs. 2C; P-value = 0.0209, 2E; P-value = 0.0007 and 2F; P-value =0.0014), as compared to the nisin variant used alone. No significant difference in bacterial inhibition was observed for nisin variants in combination with thymol (Fig. 2A; P-value =0.0681 and 2B; P-value =0.5645). Growth curves with *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} show that combinations of nisin V and thymol (100 µg ml^{-1}) or carvacrol (200 µg ml^{-1}) result in a longer lag phase than when either treatment is used singly (Figs. 3A and 3C). A similar pattern was observed when nisin S29A was used in combination with thymol or carvacrol (Figs. 3B and 3D). Indeed, statistically significant differences in

bacterial inhibition were recorded when nisin variants (V and S29A) used in combination with the essential oils thymol and carvocrol (Figs. 3A; P-value =0.0022, 3B; P-value = 0.0176, 3C; P-value =0.0029 and 3D; P-value = 0.0001), as compared to the nisin variant used alone. No significant difference in lag time was observed for nisin variants in combination with 75 µg ml^{-1} trans-cinnamaldehyde (Fig. 3E; P-value =0.9675 and 3F; P-value =0.4427). Ultimately, the most significant delay in growth was observed when nisin V and carvacrol were combined against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} (Fig. 3C). Overall, it was apparent that, in general, the use of nisin V or nisin S29A resulted in greater inhibitory effects on growth than was observed when nisin A alone was used, and this phenomenon was also apparent when the bacteriocins were used together with essential oils.

Following on from this, the bactericidal activities of nisin peptides and essential oils against the Gram negative pathogens were investigated through kill curve analysis. *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} were treated respectively with 60 µM and 30 µM of each nisin peptide in combination with 150 µg ml^{-1} thymol, 300 µg ml^{-1} carvacrol or 350 µg ml^{-1} trans-cinnamaldehyde (Figs. 4 and 5). In general, an approximate 1-log reduction in pathogen cell numbers were observed when either nisin peptides or essential oils were used alone, with the exception that carvacrol and trans-cinnamaldehyde had no effect against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (Figs. 4B and 4C, respectively) and the use of thymol alone against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} resulted in an approximate 3.5-log reduction in cell numbers (Fig. 5A). The killing effect of nisin peptides was more marked by the addition of essential oils. The antimicrobial activity of nisin peptides was significantly enhanced when used in combination with

thymol (~2-log reduction), carvacrol (~4-log reduction) and trans-cinnamaldehyde (~1.5-log reduction) against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (Fig. 4). A similar significant level of inactivation of *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} was achieved when thymol (~4.5-log reduction), carvacrol (~3-log reduction) and trans-cinnamaldehyde (~2-log reduction) were used in combination with nisin peptides (Fig. 5). It was also established that the use of bioengineered nisin derivatives in combination with essential oils was more effective than nisin A-essential oil combinations. A significantly greater reduction in *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} was observed when thymol was used in combination with nisin V or nisin S29A, rather than nisin A ($P<0.05$) (Fig. 5A). A similar observation was made when trans-cinnamaldehyde and *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} were utilised, in that a considerable reduction in viable cell numbers was observed when the essential oil was used in conjunction with nisin V (Fig. 4C). Furthermore, an increased bactericidal activity was seen when combinations of nisin V and nisin S29A with carvacrol were used, resulting in a 2-log greater reduction in *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} cell counts than was the case when a nisin A-carvacrol combination was used (Fig. 4B).

3.4 Model food trials

Having shown the enhanced potency of bioengineered nisin variants in combination with carvacrol against both Gram negative pathogens using kill curve assays, this essential oil was selected for further investigation in model food systems. To this end, commercially available powdered infant formula milk and commercially

produced apple juice were chosen as they have been associated with disease outbreaks of *C. sakazakii* and *E. coli* O157:H7, respectively. For the powdered infant formula milk studies, nisin A, nisin V or nisin S29A (60 µM) were added alone or in combination with carvacrol (300 µg ml^{-1}) (Fig. 6A). The addition of carvacrol did not significantly alter the pH. The milk was subsequently spiked with *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} at a concentration of 1×10^5 cfu ml^{-1} and incubated at room temperature for 3 h. Serial dilution and subsequent plate counts revealed that nisin and/or essential oil treatment had no effect on *C. sakazakii* NCTC 8155::p16S*lux*-P_{help}, as cell numbers remained unaltered (Fig 6A). It was noted, however, that the addition of the food-grade antimicrobial, citric acid (30 mM) reduced viable cell numbers of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (~0.5-log reduction) while combinations of nisin and citric acid resulted in a significant decrease of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} cell numbers (1-log reduction) compared to nisin usage alone ($P<0.001$) (Fig. 6B). Combinations of all three antimicrobials improved antibacterial activity relative to nisin and the essential oil carvacrol. While there was a numerical improvement in activity relative to that of citric acid, this was not significant (Fig. 6C). Additionally, nisin variant combinations did not display an enhanced potency compared to the equivalent nisin A combined treatment.

Given that the essential oil/nisin combinations did not significantly enhance the efficacy of citric acid against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in infant formula, we investigated the possibility that another nisin-based, food grade formulation could prove to be effective. For this purpose Nisaplin, a commercial formulation containing nisin A was used together with citric acid. Notably, this

405 combination brought about a significant >3-log reduction in bacterial cell counts (Fig.
406 7). This effect was not further augmented through the addition of carvacrol.

407 The commercially produced apple juice was filtered and the pH measured.
408 Nisin A, nisin V or nisin S29A (30 µM) was added to the juice alone or in combination
409 with low level of carvacrol (75 µg ml^{-1}) before *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}
410 was introduced at a concentration of 1×10^5 cfu ml^{-1}. Following incubation for 3 h at
411 room temperature, serial dilutions and plate counts on Sorbitol MacConkey agar
412 were carried out to enumerate bacterial cell counts. When nisin A, nisin V, nisin S29A
413 or carvacrol were used alone, an approximate 1-log reduction in *E. coli* O157:H7 TUV
414 93-0::p16S*lux*-P_{help} cell numbers were obtained. The effectiveness of nisin A was
415 significantly improved when used in conjunction with carvacrol, resulting in a 2.5-log
416 reduction in cells counts compared to that of the initial inoculum. Notably, the
417 combinations of nisin V and nisin S29A with carvacrol were even more effective in
418 that a 3-log reduction in bacterial cell counts was attained over the 3 h period (Fig.
419 8). More importantly, these results demonstrate that the enhanced effectiveness of
420 nisin variants observed using laboratory-based assays are retained and can be
421 translated to food systems.

422

423 **4. Discussion**

424

425 It has previously been reported that the phenolic compounds carvacrol and
426 thymol have the ability to degrade the outer membranes of the Gram negative
427 bacteria, *E. coli* and *Salmonella Typhimurium* (Helander et al., 1998). This
428 phenomenon likely explains why exposure to these compounds increases the

sensitivity of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} to nisin. Our investigations also highlight the enhanced potency of nisin when combined with trans-cinnamaldehyde. In the latter case, the mechanism involved may be different as trans-cinnamaldehyde does not disintegrate the outer membrane like carvacrol and thymol, but is believed that at sub-inhibitory concentrations, inhibits the activity of trans-membrane ATPase (Gill and Holley, 2006a, b).

This study also demonstrates the frequently superior activity of bioengineered nisin variants over their wild type nisin A equivalent when used in combination with a variety of essential oils. In growth curve assays, nisin V-essential oil and nisin S29A-esssential oil combinations, except when combined with trans-cinnamaldehyde against *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}, surpassed the activity of their nisin A counterparts as observed by the longer delay in growth. With respect to time-kill assays, nisin variant-combinations outperformed their corresponding nisin A-combinations by at least 1-log reduction in cell numbers against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (carvacrol and trans-cinnamaldehyde) and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} (thymol and carvacrol). This increased effectiveness did not translate into powdered infant formula milk (Fig. 6A). It is known that the greater availability of nutrients in foods compared to laboratory media may enable bacterial cells to repair damaged cells faster (Gill et al., 2002). As a consequence of this protective nature, a greater concentration (approximately two-fold) of essential oils are required to achieve the same effect in foods, such as semi-skimmed milk (Karatzas et al., 2001). Instead of increasing concentrations of carvacrol and potentially altering the sensory properties of the infant milk formula,

453 the preservative citric acid (30 mM) was incorporated. A significant reduction (1-log)
454 in bacterial counts was observed when nisin and citric acid were used simultaneously
455 compared to nisin alone (Fig. 6B). However, the combination of all three
456 antimicrobial agents did not improve significantly on the activity of citric acid against
457 *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} (Fig. 6C). The substitution of nisin peptides for
458 food-grade Nisaplin (10 mg ml^{-1}) proved most effective as >3-log reduction in *C.*
459 *sakazakii* NCTC 8155::p16S*lux*-P_{help} cell counts was observed after treatment with
460 Nisaplin and citric acid (Fig. 7). Nisaplin contains 2.5% active nisin and the remainder
461 is made up of a balance of sodium chloride and denatured milk solids. The high
462 percentage of salts present is likely to contribute to antimicrobial activity. The nisin-
463 containing formulation was employed as it is commercially available and approved
464 for use in over 50 countries worldwide. Should equivalent forms of nisin V/S29A be
465 generated, there is the potential for these peptides to be used in the same way with
466 the possibility of even greater antimicrobial efficacy.

467 Due to the demand for minimally processed foods, researchers have
468 previously investigated the use of natural antimicrobials including nisin and
469 cinnamon in apple juice (Yuste and Fung, 2004). In this study, we also investigated
470 the merits of using nisin in combination with the essential oil carvacrol in apple juice.
471 The combination of the bioengineered nisin variants with carvacrol accelerated
472 bacterial death, resulting in a >3-log reduction in *E. coli* O157:H7 TUV 93-0::p16S*lux*-
473 P_{help} cell counts after 3 h (Fig. 8). The low pH of the apple juice may partially
474 contribute to this effect. It is also known that essential oils are most effective at
475 acidic pHs (Burt, 2004) due to the increase in hydrophobicity and better diffusion
476 into the lipid phase of the membrane (Juven et al., 1994). Moreover, nisin is more

stable at acidic pH and therefore more effective (Delves-Broughton, 1990). While, the manufacture of fruit juices should include effective treatments so as to result in a cumulative 5-log reduction in the numbers of *E. coli* O157:H7 as specified by the Food and Drug Administration's Guidance for Industry (FDA, 2001), the detection limit in this study was 10^2 cfu ml^{-1} and thus the use of more sensitive detection methods may reveal that this cumulative reduction can be achieved.

The use of essential oils in foods has been limited due to the high concentrations required to achieve sufficient antimicrobial activity (Hyldgaard et al., 2012). Notably, however, this study shows that relatively low concentrations of carvacrol (approximately 0.0075%) could be used in combination with nisin to inhibit *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help} in apple juice. Before commercial application, sensory studies would have to be carried out to determine the organoleptic properties of apple juice with essential oils. Although, upon addition of such a low concentration of carvacrol in this study, no adverse aroma was observed. Recently, sensory evaluations were carried out to assess the consequence of adding 75 µl L^{-1} (~63.75 µg ml^{-1}) of lemon essential oil to apple juice. Researchers found that this relatively small concentration of essential oil did not decrease the acceptability of the sample or the organoleptic properties (Espina et al., 2012). Moreover, all of the tested essential oils have GRAS status by both the EU and the FDA (EU, 2012; FDA, Revised 2014a, b) meaning that their addition could be permitted if they were used in the minimum quantities and with good manufacturing processes.

5. Conclusion

The combination of nisin peptides and essential oils could pave the way for new hurdle concepts when it comes to food preservation, in particular towards Gram negative bacteria. Moreover, the combinatory effects could lead to reduced treatment intensity and/or antimicrobial dosage and therefore avoid undesirable sensory and nutritional properties in food. Such combinations could enhance food safety, shelf life and quality while also meeting consumer demands for more natural, preservative-free foods.

Acknowledgements

This work was carried out through a PhD Programme in Molecular Cell Biology funded by the Programme for Research in Third-Level Institutions (PRTLI) awarded to AC. Work in the authors' laboratory is supported by the Irish Government under the National Development Plan, through Science Foundation Ireland Investigator awards (10/ IN.1/B3027) and (06/IN.1/B98) (http://www.sfi.ie). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

We thank Paula O'Connor for mass spectrometry and Paddy O'Reilly for technical assistance.

References

Bierbaum, G., Sahl, H.G., 2009. Lantibiotics: mode of action, biosynthesis and bioengineering. Curr Pharm Biotechnol 10, 2-18.

524

525 Bowen, A.B., Braden, C.R., 2006. Invasive *Enterobacter sakazakii* disease in infants.
526 Emerg Infect Dis 12, 1185-1189.

527

528 CDC, 2002. *Enterobacter sakazakii* infections associated with the use of powdered
529 infant formula--Tennessee, 2001. MMWR Morb Mortal Wkly Rep 51, 297-300.

530

531 Cotter, P.D., Hill, C., Ross, R.P., 2005. Bacteriocins: developing innate immunity for
532 food. Nat Rev Microbiol 3, 777-788.

533

534 Delves-Broughton, J., 1990. Nisin and its application as a food preservative.
535 International Journal of Dairy Technology 43, 73-76.

536

537 Drudy, D., Mullane, N.R., Quinn, T., Wall, P.G., Fanning, S., 2006. *Enterobacter*
538 *sakazakii*: an emerging pathogen in powdered infant formula. Clin Infect Dis 42, 996-
539 1002.

540

541 Espina, L., Somolinos, M., Ouazzou, A.A., Condon, S., Garcia-Gonzalo, D., Pagan, R.,
542 2012. Inactivation of *Escherichia coli* O157:H7 in fruit juices by combined treatments
543 of citrus fruit essential oils and heat. Int J Food Microbiol 159, 9-16.

544

545 Ettayebi, K., El Yamani, J., Rossi-Hassani, B., 2000. Synergistic effects of nisin and
546 thymol on antimicrobial activities in *Listeria monocytogenes* and *Bacillus subtilis*.
547 FEMS Microbiol Lett 183, 191-195.

EU, 2012. Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. Official Journal of the European Union L267, 1-161.

FDA, 2001. Hazard Analysis and Critical Control Point (HACCP); Procedures for the Safe and Sanitary Processing and Importing of Juice: Final Rule. U.S. Food and Drug Administration, Washington, D.C. Federal Register.

FDA, Revised 2014a. Code of Federal Regulations Title 21, Food additives permitted for direct addition to food for human consumption; flavouring agents and related substances; synthetic flavouring substances and adjuvants. U.S. Food and Drug Administration, Washington, D.C.

FDA, Revised 2014b. Code of Federal Regulations Title 21, Substances generally recognized as safe; General Provisions; Synthetic flavouring substances and adjuvants. U.S. Food and Drug Administration, Washington, D.C.

Field, D., Daly, K., O'Connor, P.M., Cotter, P.D., Hill, C., Ross, R.P., 2015a. Efficacies of nisin A and nisin V semipurified preparations alone and in combination with plant

essential oils for controlling *Listeria monocytogenes*. Appl Environ Microbiol 81, 2762-2769.

Field, D., Cotter, P.D., Ross, R.P., Hill, C., 2015b. Bioengineering of the model lantibiotic nisin. Bioengineered 6, 187-192.

Field, D., Begley, M., O'Connor, P.M., Daly, K.M., Hugenholtz, F., Cotter, P.D., Hill, C., Ross, R.P., 2012. Bioengineered nisin A derivatives with enhanced activity against both Gram positive and Gram negative pathogens. PLoS One 7, e46884.

Field, D., Quigley, L., O'Connor, P.M., Rea, M.C., Daly, K., Cotter, P.D., Hill, C., Ross, R.P., 2010. Studies with bioengineered Nisin peptides highlight the broad-spectrum potency of Nisin V. Microb Biotechnol 3, 473-486.

Field, D., Connor, P.M., Cotter, P.D., Hill, C., Ross, R.P., 2008. The generation of nisin variants with enhanced activity against specific gram-positive pathogens. Mol Microbiol 69, 218-230.

Forsythe, S.J., 2005. *Enterobacter sakazakii* and other bacteria in powdered infant milk formula. Matern Child Nutr 1, 44-50.

Gill, A.O., Delaquis, P., Russo, P., Holley, R.A., 2002. Evaluation of antilisterial action of cilantro oil on vacuum packed ham. Int J Food Microbiol 73, 83-92.

595 Gill, A.O., Holley, R.A., 2006a. Disruption of *Escherichia coli*, *Listeria monocytogenes*
596 and *Lactobacillus sakei* cellular membranes by plant oil aromatics. Int J Food
597 Microbiol 108, 1-9.
598
599 Gill, A.O., Holley, R.A., 2006b. Inhibition of membrane bound ATPases of *Escherichia*
600 *coli* and *Listeria monocytogenes* by plant oil aromatics. Int J Food Microbiol 111, 170-
601 174.
602
603 Gurtler, J.B., Kornacki, J.L., Beuchat, L.R., 2005. *Enterobacter sakazakii*: a coliform of
604 increased concern to infant health. Int J Food Microbiol 104, 1-34.
605
606 Helander, I.M., Alakomi, H.-L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J.,
607 Gorris, L.G.M., von Wright, A., 1998. Characterization of the action of selected
608 essential oil components on Gram-negative bacteria. Journal of Agricultural and
609 Food Chemistry 46, 3590-3595.
610
611 Ho, N.K., Henry, A.C., Johnson-Henry, K., Sherman, P.M., 2013. Pathogenicity, host
612 responses and implications for management of enterohemorrhagic *Escherichia coli*
613 O157:H7 infection. Can J Gastroenterol 27, 281-285.
614
615 Hyldgaard, M., Mygind, T., Meyer, R.L., 2012. Essential oils in food preservation:
616 mode of action, synergies, and interactions with food matrix components. Front
617 Microbiol 3, 12.
618

619 Iversen, C., Forsythe, S., 2004. Isolation of *Enterobacter sakazakii* and other
620 *Enterobacteriaceae* from powdered infant formula milk and related products. Food
621 Microbiology 21, 771-777.
622
623 Juven, B.J., Kanner, J., Schved, F., Weisslowicz, H., 1994. Factors that interact with
624 the antibacterial action of thyme essential oil and its active constituents. J Appl
625 Bacteriol 76, 626-631.
626
627 Karatzas, A.K., Kets, E.P., Smid, E.J., Bennik, M.H., 2001. The combined action of
628 carvacrol and high hydrostatic pressure on *Listeria monocytogenes* Scott A. J Appl
629 Microbiol 90, 463-469.
630
631 Kuipers, O.P., Beerthuyzen, M.M., Siezen, R.J., De Vos, W.M., 1993. Characterization
632 of the nisin gene cluster nisABTCIPR of *Lactococcus lactis*. Requirement of expression
633 of the nisA and nisI genes for development of immunity. Eur J Biochem 216, 281-291.
634
635 Kuipers, O.P., De Ruyter, P.G., Kleerebezem, M., De Vos, W.M., 1998. Quorum
636 sensing-controlled gene expression in lactic acid bacteria. J Biotechnol 64, 15-21.
637
638
639 Norberg, S., Stanton, C., Ross, R.P., Hill, C., Fitzgerald, G.F., Cotter, P.D., 2012.
640 *Cronobacter* spp. in powdered infant formula. J Food Prot 75, 607-620.
641

642 Olasupo, N.A., Fitzgerald, D.J., Gasson, M.J., Narbad, A., 2003. Activity of natural
643 antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovar
644 *Typhimurium*. Lett Appl Microbiol 37, 448-451.
645
646 Olasupo, N.A., Fitzgerald, D.J., Narbad, A., Gasson, M.J., 2004. Inhibition of *Bacillus*
647 *subtilis* and *Listeria innocua* by nisin in combination with some naturally occurring
648 organic compounds. J Food Prot 67, 596-600.
649
650 Periago, P.M., Moezelaar, R., 2001. Combined effect of nisin and carvacrol at
651 different pH and temperature levels on the viability of different strains of *Bacillus*
652 *cereus*. Int J Food Microbiol 68, 141-148.
653
654 Pol, I.E., Smid, E.J., 1999. Combined action of nisin and carvacrol on *Bacillus cereus*
655 and *Listeria monocytogenes*. Lett Appl Microbiol 29, 166-170.
656
657 Sahl, H.G., Jack, R.W., Bierbaum, G., 1995. Biosynthesis and biological activities of
658 lantibiotics with unique post-translational modifications. Eur J Biochem 230, 827-
659 853.
660
661 Sobrino-López, A., Martín-Belloso, O., 2008. Use of nisin and other bacteriocins for
662 preservation of dairy products. International Dairy Journal 18, 329-343.
663

664 Stevens, K.A., Sheldon, B.W., Klapes, N.A., Klaenhammer, T.R., 1991. Nisin treatment
665 for inactivation of *Salmonella* species and other gram-negative bacteria. Appl
666 Environ Microbiol 57, 3613-3615.
667
668 Vidovic, S., Korber, D.R., 2014. *Escherichia coli* O157: Insights into the adaptive stress
669 physiology and the influence of stressors on epidemiology and ecology of this human
670 pathogen. Crit Rev Microbiol.
671
672 Wiedemann, I., Benz, R., Sahl, H.G., 2004. Lipid II-mediated pore formation by the
673 peptide antibiotic nisin: a black lipid membrane study. J Bacteriol 186, 3259-3261.
674
675 Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O.P., Bierbaum, G., de Kruijff, B.,
676 Sahl, H.G., 2001. Specific binding of nisin to the peptidoglycan precursor lipid II
677 combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic
678 activity. J Biol Chem 276, 1772-1779.
679
680 Yuste, J., Fung, D.Y., 2004. Inactivation of *Salmonella typhimurium* and *Escherichia*
681 *coli* O157:H7 in apple juice by a combination of nisin and cinnamon. J Food Prot 67,
682 371-377.
683
684
685
686
687

688

689

690

691

692

693

694

695

696

697

698

699 **Tables**

700

Strains/Lux-tagged bacteria	Relevant characteristics or source of strains	Reference
Lactococcus lactis NZ9700	Wild-type nisin producer	(Kuipers et al., 1993; Kuipers et al., 1998)
Lactococcus lactis NZ9800	*L. lactis* NZ9700ΔnisA	(Kuipers et al., 1993; Kuipers et al., 1998)
Lactococcus lactis NZ9800 pCI372*nisA*	Wild-type nisin-producing strain	(Field et al., 2008)
Lactococcus lactis NZ9800 pCI372*nisA*::M21V	Wild-type producing strain + alteration at position 21	(Field et al., 2010)
Lactococcus lactis NZ9800 pCI372*nisA*::S29A	Wild-type nisin-producing strain + alteration at position 29	(Field et al., 2012)
Cronobacter sakazakii NCTC 8155::p16S*lux*-P_{help}	Isolated from dried milk powder and transformed with p16Slux-P_{help} plasmid	UCC Culture Collection
Escherichia coli O157:H7 TUV 93-0::p16S*lux*-P_{help}	Derived from strain EDL933 and transformed with p16Slux-P_{help}	UCC Culture Collection

701

702 **Table 1**

List of nisin-producing strains and *lux*-tagged bacterial strains used in this study, including relevant characteristics and references.

Indicator organism	Nisin A µg ml^{-1} (µM)	Nisin V µg ml^{-1} (µM)	Nisin S29A µg ml^{-1} (µM)	Thymol µg ml^{-1}	Carvacrol µg ml^{-1}	Cinnamalde-hyde µg ml^{-1}
C. sakazakii NCTC 8155::p16S*lux*-P_{help}	12.57 (3.75)	12.57 (3.75)	**6.28 (1.875) (P<0.001)**	125	250	250
E. coli O157:H7 TUV 93-0::p16S*lux*-P_{help}	25.14 (7.5)	**12.57 (3.75) (P<0.001)**	**12.57 (3.75) (P<0.001)**	250	250	250

Table 2

Minimum inhibitory concentration determinations of nisin A, nisin V, nisin S29A and the essential oils thymol, carvacrol and trans-cinnamaldehyde against *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} and *E. coli* O157:H7 TUV 93-0::p16S*lux*-P_{help}. Results are expressed as the mean of triplicate assays. Values in bold represent statistical difference between nisin variants and wild type nisin A (Student's t-test: P<0.05).

Figure legends

Fig. 1. Structure and mass spectrometry analysis of nisin A and its derivatives. **(A)** Structural composition of nisin A. Blue circles indicate where amino acids were altered resulting in the generation of nisin variants with enhanced activity.

Figure 2. Growth curve analysis of *C. sakazakii* NCTC8155::p16S*lux*-P_{help} with 30µM nisin A (open square), **(A)** nisin V (open diamond), 100µg/ml thymol (open circle) and combinations of nisin A and thymol (closed square), nisin V and thymol (closed diamond) **(B)** nisin S29A (open inverted triangle), 100µg/ml thymol (open circle) and combinations of nisin A and thymol (closed square), nisin S29A and thymol (closed

747 inverted triangle). **(C)** nisin V (open diamond), 125μg/ml carvacrol (open circle) and
748 combinations of nisin A and carvacrol (closed square), nisin V and carvacrol (closed
749 diamond) **(D)** nisin V (open diamond), nisin S29A (open inverted triangle), 125μg/ml
750 carvacrol (open circle) and combinations of nisin A and carvacrol (closed square) and
751 nisin S29A and thymol (closed inverted triangle). **(E)** nisin V (open diamond),
752 125μg/ml trans-cinnamaldehyde (open circle) and combinations of nisin A and
753 trans-cinnamaldehyde (closed square), nisin V and trans-cinnamaldehyde (closed
754 diamond) **(F)** nisin S29A (open inverted triangle), 125μg/ml trans-cinnamaldehyde
755 (open circle) and combinations of nisin A and trans-cinnamaldehyde (closed
756 square)and nisin S29A and trans-cinnamaldehyde (closed inverted triangle).
757
758 **Figure 3.** Growth curve analysis of *E. coli* O157:H7 TUV93-0::p16S*lux*-P_{help} with 30μM
759 nisin A (open square), **(A)** nisin V (open diamond), 100μg/ml thymol (open circle) and
760 combinations of nisin A and thymol (closed square), nisin V and thymol (closed
761 diamond) **(B)** nisin S29A (open inverted triangle), 100μg/ml thymol (open circle) and
762 combinations of nisin A and thymol (closed square), nisin S29A and thymol (closed
763 inverted triangle). **(C)** nisin V (open diamond), 125μg/ml carvacrol (open circle) and
764 combinations of nisin A and carvacrol (closed square), nisin V and carvacrol (closed
765 diamond) **(D)** nisin V (open diamond), nisin S29A (open inverted triangle), 125μg/ml
766 carvacrol (open circle) and combinations of nisin A and carvacrol (closed square) and
767 nisin S29A and thymol (closed inverted triangle). **(E)** nisin V (open diamond),
768 125μg/ml trans-cinnamaldehyde (open circle) and combinations of nisin A and
769 trans-cinnamaldehyde (closed square), nisin V and trans-cinnamaldehyde (closed
770 diamond) **(F)** nisin S29A (open inverted triangle), 125μg/ml trans-cinnamaldehyde

771 (open circle) and combinations of nisin A and trans-cinnamaldehyde (closed
772 square)and nisin S29A and trans-cinnamaldehyde (closed inverted triangle).
773
774 **Fig. 4.** Kill effect of nisin A derivatives in combination with essential oils against *C.*
775 *sakazakii* NCTC 8155::p16S*lux*-P_{help}. Kill curve analysis of *C. sakazakii* NCTC
776 8155::p16S*lux*-P_{help} with 60 µM of each peptide both alone and in combination with
777 **(A)** 150 µg ml^{-1} thymol (THY), **(B)** 300 µg ml^{-1} carvacrol (CA) and **(C)** 350 µg ml^{-1} trans-
778 cinnamaldehyde (CN) in LB broth after 3 hours at room temperature.
779
780 **Fig. 5.** Kill effect of nisin A derivatives in combination with essential oils against *E. coli*
781 O157:H7 TUV 93-0::p16S*lux*-P_{help}. Kill curve analysis of *E. coli* O157:H7 TUV 93-
782 0::p16S*lux*-P_{help} with 30 µM of each nisin peptide both alone and in combination with
783 **(A)** 150 µg ml^{-1} thymol (THY), **(B)** 300 µg ml^{-1} carvacrol (CA) and **(C)** 350 µg ml^{-1} trans-
784 cinnamaldehyde (CN) in LB broth after 3 hours at room temperature. ND, not
785 detected.
786
787 **Fig. 6.** Combinations of nisin derivatives, carvacrol and citric acid against *C. sakazakii*
788 NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula. Model food analysis of *C.*
789 *sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula with 60 µM of
790 each nisin peptide both alone and in combination with **(A)** 300 µg ml^{-1} carvacrol (CA),
791 **(B)** 30 mM citric acid (CT) and **(C)** 300 µg ml^{-1} carvacrol and 30 mM citric acid after 3
792 hours at room temperature.
793

794 **Fig. 7.** Combinations of commercial Nisaplin, carvacrol and citric acid against *C.*
795 *sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula. Model food
796 analysis of *C. sakazakii* NCTC 8155::p16S*lux*-P_{help} in powdered infant milk formula
797 with 10 mg ml^{-1} of commercial Nisaplin (Sigma) containing 2.5% nisin both alone and
798 in combination with 300 µg ml^{-1} carvacrol (CA) and 30 mM citric acid (CT) after 3
799 hours at room temperature. ND, not detected (detection limit of 10^2 cfu ml^{-1}).
800
801
802 **Fig. 8.** Combinations of nisin A derivatives and carvacrol against *E. coli* O157:H7 TUV
803 93-0::p16S*lux*-P_{help} in apple juice. Model food analysis of *E. coli* O157:H7 TUV 93-
804 0::p16S*lux*-P_{help} in apple juice with 30 µM of each nisin peptide both alone and in
805 combination with 75 µg ml^{-1} carvacrol (CA) after 3 hours at room temperature. ND,
806 not detected (detection limit of 10^2 cfu ml^{-1}).
807
808